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Extrachromosomal DNA in the Apicomplexa

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INTRODUCTION: TWO EXTRACHROMOSOMAL GENOMES

Many microbiologists are unfamiliar with the diverse array of unicellular protozoa known as apicomplexans (phylum Apicomplexa, Levine 1970), even though this group includes that most devastating human pathogen, the malaria parasite (genus *Plasmodium*), the AIDS-related opportunistic pathogens *Toxoplasma* and *Cryptosporidium*, and the economically important pathogens of cattle and chickens, *Theileria* and *Eimeria*. Other members of the group are numerous and widely distributed, often on marine organisms (68). All members of the phylum are obligate intracellular parasites, and they are named after a set of specialized organelles, the apical complex, which they use in attaching to or penetrating host cells. Classical genetic studies have been limited by the intracellular habits and complex life cycles of these organisms, but the development of pulsed-field gel electrophoresis, DNA sequencing, and related techniques like PCR has allowed rapid progress in understanding the nuclear genome in both physical and genetic terms. In addition, the recent advent of transfection techniques for apicomplexans is likely to lead to an exponential increase in our knowledge of the molecular biology of these parasites.

One aspect of their existence that has only recently begun to yield answers to long-standing questions is the nature and function of their DNA-containing organelles. The specialized nature of the malarial mitochondrion, for instance, has been recognized for many years and has been the subject of various biochemical studies (32), but its precise role, particularly in the bloodstream of the vertebrate host, is still uncertain. About 8 years ago, however, mitochondrial DNA molecules (mtDNA) were identified for the first time in malaria parasites as repetitive 6-kb DNA sequences, and in the intervening years the genetic content of these molecules and their counterparts from *Theileria* parasites has been firmly established. As it happens, the discovery of mtDNA was pre-dated by the discovery of another organellar DNA, the so-called 35-kb circle, first wrongly supposed to be mitochondrial in origin. Subsequent work in our own laboratory has led to the surprising conclusion that this molecule is in fact the remnant of an algal plastid genome, probably acquired through secondary endosymbiosis by a progenitor of the phylum in ancient times (125). The analysis of primary sequence information from both the mitochondrial and plastid-like extrachromosomal genomes of one malarial species, *Plasmodium falciparum*, is drawing toward its completion, and so it is appropriate for this review to set out their basic organization and highlight special features against which further research on it and other apicomplexans can be gauged.

DISCOVERY: EARLY YEARS

Malaria parasites were the first apicomplexans to yield the secrets of their organellar DNAs but the road to their discovery and eventual identification was circuitous.

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The Plastid-Like Genome

The plastid-like DNA (plDNA), usually known as the 35-kb circle (Fig. 1c), was first noted in *P. knowlesi* as a low-density minor satellite in CsCl gradients (48), and its role in the cell was tentatively suggested to be mitochondrial. Chance et al. (13) made no comment on the possible origin of similar low-density satellites they found in two rodent malaria parasites, *P. berghei* and *P. chabaudi*. Kilejian (63) was the first person to "see" the 35-kb circles. In a pioneering electron microscopic study, she found them in extracts of isolated "mitochondria" of the avian parasite *P. lophurae*. They had a contour length of 10.3 μm (ca. 31 kb) and a buoyant density linking them to the previously noted satellites. She understandably assumed them to be of mitochondrial origin. (It is of historical interest that some of her preparations yielded linear molecules of various lengths and a buoyant density in CsCl of 1.79 g/cm^3. Kilejian supposed that they were contaminating fragments of nuclear DNA, but with hindsight it is conceivable that these were in fact the true mtDNA molecules, the 6-kb element, which we discuss below.) Some 8 years later, comparable circular molecules were seen in electron microscopic preparations of organellar DNA from *P. berghei* (20), and yet again they were assigned a mitochondrial origin. The circle was next spotted in the DNA of another apicomplexan, *Toxoplasma gondii* (10). Following the trend of earlier observers, these authors supposed the 12-μm circles to be mitochondrial but made the insightful suggestion that a large cruciform structure might reflect the presence of an inverted repeat of rRNA genes, a feature rarely found in mtDNAs. As we discuss below, the presence in *Toxoplasma* of this homolog of the malarial circle and its inverted rDNA repeat has since been amply confirmed.

The circular molecules were first isolated in usable quantity from the simian parasite *P. knowlesi* (120). They were 11.5 μm in contour length and showed a cruciform structure just like that of *Toxoplasma*. Gardner et al. (35) isolated the counterpart from the human parasite *P. falciparum* and substantiated the organellar origin of the molecules by obtaining a sequence corresponding to a fragment of a prokaryotic small-subunit (SSU) rRNA gene. Not surprisingly, the molecule was once more assumed to be mitochondrial, as it was by Feagin et al. (28). However, as discussed below, the circle is almost certainly packaged in a different organelle.

The Mitochondrial DNA

The discovery of repetitive 6-kb DNA sequences in the rodent pathogen *P. yoelii*, which were conserved across a variety of malaria parasites (108), led to the start of a new era. Initially, these tandem sequences were regarded as repetitive elements of chromosomes, but sequence information from *P. yoelii* (107) and the avian parasite *P. gallinaceum* (2) revealed that the so-called 6-kb element carried genes for the characteristically mitochondrial proteins, cytochrome *b* (*cyt b*) and subunit I of cytochrome *c* oxidase (*cox* I). This raised two problems: at 6 kb, the element seemed far too small to constitute a complete mitochondrial genome, and in any case, that slot had already been allocated to the 35-kb circle. Perhaps the 6-kb element was a mitochondrial episome or a partner in a bipartite mitochondrial genome (28, 61).

This dilemma began to be resolved when additional sequence information from the 35-kb circle pointed to its plastid-like provenance (40). Even though only the plDNA has been definitively assigned to an organelle (see below), accumulating information about the primary sequence, replication, and transcription of the two DNA species has clearly shown them to be conceptually distinct entities, in terms of both their evolution and their structure. No candidate for a mitochondrial partner for the 6-kb element has been found, and it has come to be accepted that the 6-kb element constitutes the entire mitochondrial genome of the malaria parasite while, as discussed below, homologous sequences have been found in other apicomplexans.

COPY NUMBERS, ORGANELLES, AND INHERITANCE

The 6-kb element is present in a moderately high copy number, with estimates varying between 15 per cell in *P. gallinaceum* (61), 20 per cell in *P. falciparum* (87), and 150 per cell in *P. yoelii* (108). The cytoplasmic location of these molecules is clearly indicated by their pattern of inheritance in sexual reproduction: by analyzing individual oocysts (effectively the meiotic progeny of single zygotes) from a *P. falciparum* cross between parents with 6-kb elements differing at a particular nucleotide, inheritance was shown to be uniparental (18), while analysis of gametocytes from *P. gallinaceum* indicated that the macrogamete (female) was the transmitting parent (17, 110). It is noteworthy that mitochondria occur in both macro- and microgametes in *Toxoplasma* and *Eimeria* (96), but the inheritance of mitochondrial markers in these cases has not been studied.

As discussed in the next section, the identity and arrangement of the genes on the 35-kb circle overwhelmingly favored its evolution from a plastid genome, and, as with mtDNA, its presumed organellar location was supported by its uniparental inheritance via macrogametes in sexual reproduction (17, 110). Analysis of subcellular fractions from *P. yoelii* indicated that the organelle carrying the 35-kb circles did not cosegregate with the mitochondria (123), and it has been speculated (64, 121) that the so-called spherical body of the malaria parasite, (1), an inconspicuous object of unknown function (going by a variety of names in other apicomplexans, e.g., the hohlzylinder or Golgi adjunct of *Toxoplasma* [Fig. 1b]), might be the organelle in question (98). This location has since been confirmed in *T. gondii* (72).

The vestigial plastid organelle containing the 35-kb DNA must have a mechanism for ensuring accurate segregation during schizogony, since copy number estimates based on Southern blot analyses have indicated that there are only one or two 35-kb circles per cell in ring stage developmental forms of *P. falciparum* (85a). A somewhat higher copy number (ca. 8) was found in *T. gondii* (91a), but nothing is known about the segregation mechanism, and the replication of the plDNA has not been extensively explored. It is known, however, that these molecules replicate at or just before the onset of schizogony (87, 99), and replication bubbles have been observed in the plDNA of both *P. falciparum* and *P. knowlesi* (119). Replication of mtDNA is much better understood and is discussed later in this review.

THE PLASTID-LIKE GENOME

Map and Sequence

We have recently mapped and completely sequenced the plDNA from *P. falciparum* (124). As discussed below, it appears that the homologous circular DNA in other apicomplexans has a similar organization. The map in Fig. 2 shows that the circle is packed with genes; these are usually separated by only a few nucleotides and in some cases even have small overlaps. The sequences of most of the proteins encoded by the malarial circle have diverged considerably from their counterparts in prokaryotes and other plastids. This is associated

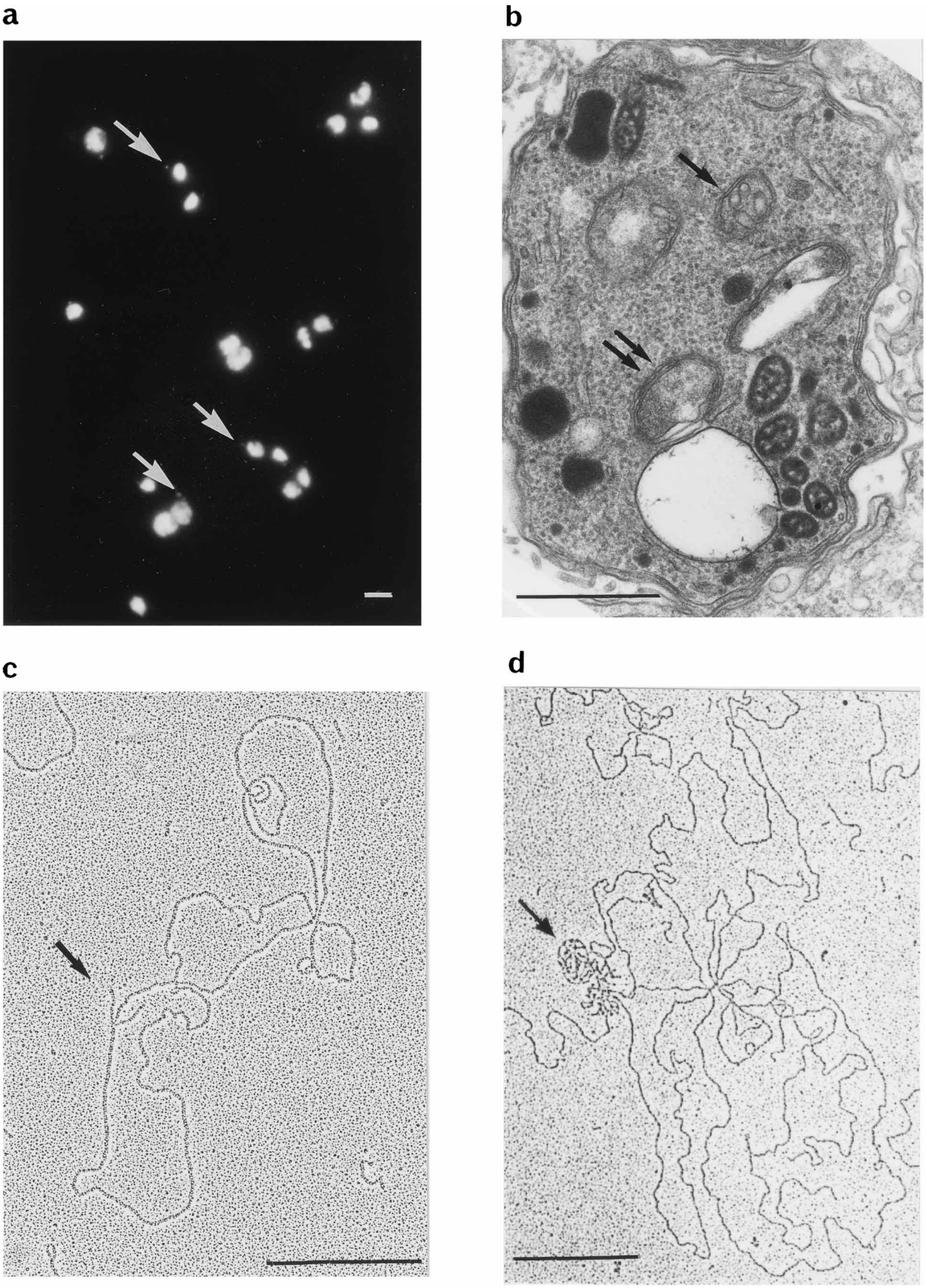

FIG. 1. Apicomplexan organelles and extrachromosomal DNA. (a) Extrachromosomal DNA (arrows) and nuclear DNA of *P. falciparum* trophozoites stained with 4′,6-diamidino-2-phenylindole (DAPI). Bar, 1 μm. (b) Electron micrographic section through the tubular mitochondrion (arrow) and putative plastid (double arrow) of a *T. gondii* tachyzoite. Bar, 1 μm. (c) 35-kb plDNA circle of *P. falciparum* with incipient cruciform (arrow). Bar, 1 μm. (d) Complex replicating mtDNA molecule from *P. falciparum* (87). The arrow indicates a single-stranded region. Bar, 1 μm.

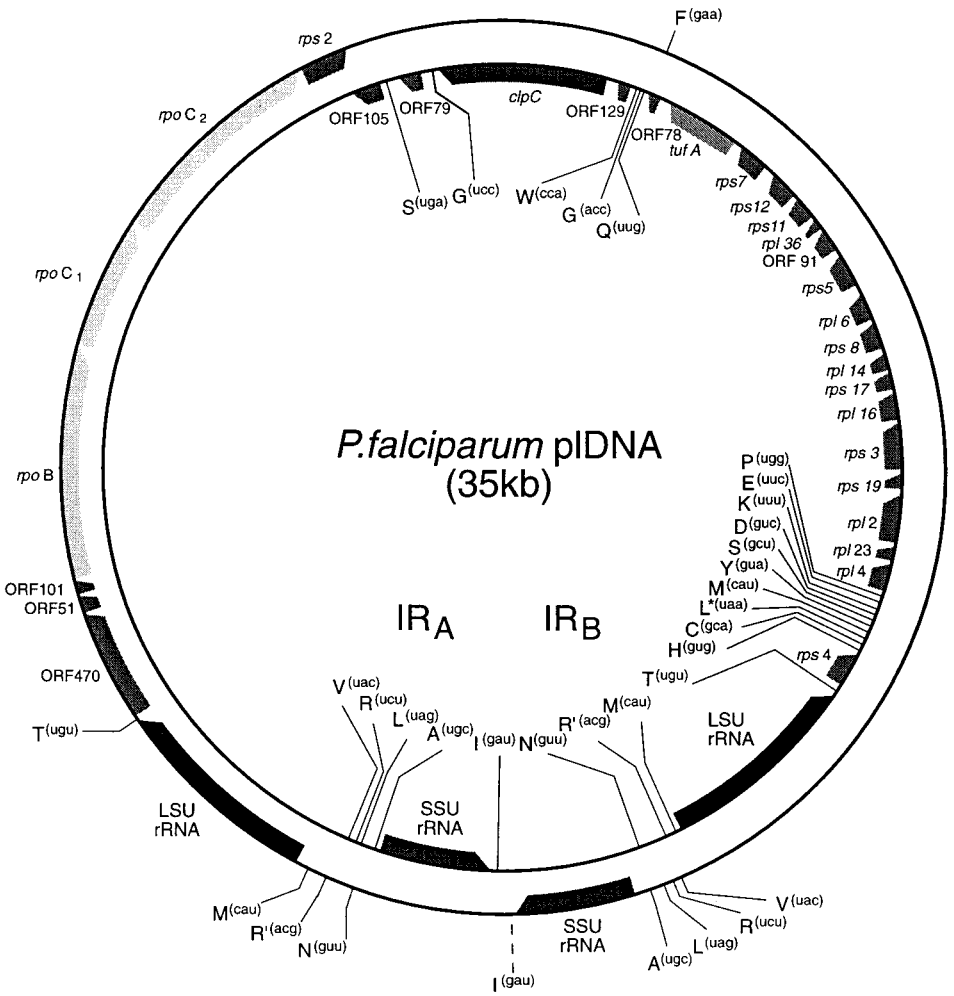

FIG. 2. Gene map of the 35-kb circular DNA of *P. falciparum*. The two sectors (A and B) of the inverted repeat (IR) are indicated, and tRNA genes are signified by both the single-letter amino acid code and anticodon. The direction of transcription is anticlockwise (inner circle) and clockwise (outer circle). Unidentified ORFs are numbered according to the length of the predicted peptide. *rpl* and *rps* encode LSU and SSU ribosomal proteins, respectively; *rpo* encodes RNA polymerase; *tuf* encodes translation elongation factor; *clp* encodes the chaperone protein. For further explanation, see the text. The full sequence has been submitted to the EMBL database with the accession numbers X95275 and X95276. Reprinted from reference 124 with permission.

with considerable bias arising from the comparatively high A+T content of the circle (86%) compared with plastids from other sources, which often has tended to obscure similarity with other genes in the database. Consequently, gene identification has depended a lot on recognizing conserved motifs, usually at a low level of homology, often coupled with clues from the arrangement of the genes on the circle. Readers should consult the map in Fig. 2 to follow the description given in the next section.

Inverted Repeat

The inverted repeat (IR) has a total length of ca. 10.5 kb and encodes duplicated genes for SSU and large-subunit (LSU) rRNAs and nine different tRNAs. One gene of each rRNA has been sequenced, but restriction fragment duplication has made it difficult for us to determine from which sector of the IR either sequence comes. Secondary-structure diagrams of the rRNAs (36, 37) were built around conserved stems and loops identified as core structures in earlier studies of rRNA (75), but there is no experimental evidence at present to support these putative structures. Comparison of the conserved regions with corresponding sequences on the 6-kb element clearly showed that the plDNA and mtDNA are not closely related (29). This helped to dispel lingering thoughts about the possibility of a complex mitochondrial genome (28). However, initial phylogenetic analysis of the rRNA genes from the plDNA did not conclusively indicate their plastid rather than mitochondrial origin, despite the presence of a signature sequence for plastid rRNA towards the 3′ end of the SSU gene (36, 37). As all the standard mathematical models for phylogenetic comparison assume that the rates of nucleotide substitutions are uniform across the lineages under scrutiny (69, 82), the most likely source of difficulty in our phylogenetic studies was the extreme A+T content of the malarial sequences. In support of this supposition, an analysis based solely on transversions, less likely to be influenced by base composition than transitions, placed the malarial SSU rRNA sequence with those of other plastids (106a). Use of the LogDet transformation to minimize the effects of nucleotide bias has led to the same conclusion (21).

As shown in Fig. 3, tRNA genes flank both forms of the rRNA gene in the IR. Restriction digests with *Sty*I, which has sites asymmetrically placed with respect to the IR, allowed us

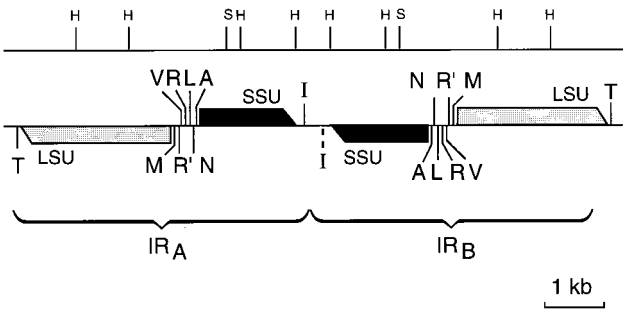

FIG. 3. Schematic of the IR region of the plDNA of *P. falciparum*. IR_A and IR_B are the two sectors of the inverted repeat as indicated in Fig. 2. tRNA genes are denoted by the conventional single-letter code, with R′ and R being nonidentical copies of trn^R; the dotted line indicates the position of a second assumed copy of trn^I, not determined by sequencing. Transcription is to the right (upper side) and to the left (lower side). Positions of *Hin*dIII (H) and *Sty*I (S) restriction sites are indicated.

to isolate separately each cluster of tRNA genes (*met* [M], *arg* [R], *val* [V], *arg′* [R′], *leu* [L], *asp* [N], and *ala* [A]) from individual arms of the palindrome, and the sequences of the duplicated forms were found to be identical (39). It is likely that the trn^I gene at the center of the IR also is duplicated, but we have not been able to sequence through the entire small *Hin*dIII fragment at the center of the palindrome to confirm this. We estimate that if there is a unique region here corresponding to the small single-copy region in typical plastid genomes, it is only tens of nucleotides in length. The IR extends for 36 nucleotides (nt) beyond the trn^T genes just downstream of the LSU rRNA genes and includes the first three codons of two different open reading frames (ORF470 and *rps* 4). We presume that the 5′ end of at least one of these ORFs is under positive selective pressure (see "Fossilized or functional" below).

The disposition of the rRNA and *trn* genes in the IR differs in several respects from that of other recorded plastids. However, this order is conserved in *T. gondii* (19a, 122), suggesting that it may be an ancestral apicomplexan character derived from numerous rearrangements and deletions in an ancient progenitor of the apicomplexan clade.

Single-Copy Region (IR_A Sector)

Immediately downstream of the inverted repeat on the IR_A sector and encoded on the same DNA strand as the LSU rRNA and trn^T lie three unassigned ORFs (ORF470, ORF101, and ORF51). We have identified a homolog in databases only for ORF470. This ORF corresponds to a highly conserved bacterial and plastid protein of unknown function, the coding region for the C terminus first being recorded in the plDNA of the red alga *Antithamnion* spp. (65). Three further unpublished sequences of plastid homologs have been made available to us, two from red algae, *Porphyra purpurea* (89a) and *Cyanidium* (*Galdiera*) *caldarum* (127), and one from the chrysophyte (diatom) *Odontella sinensis* (65a). At the predicted amino acid level, the identity of these sequences with the malarial gene ranges from 47 to 52%, giving powerful support to the notion of a plastid origin of the malarial circle (118). Because of the absence of other algal plastid sequences in the database, the possibility cannot be excluded that this ORF will be found in other forms of algae, e.g., chlorophytes, but it is not encoded in the plastid genome of either higher plants or the primitive land plant *Marchantia polymorpha* (76). However, a high level of similarity to an ORF in the cyanobacterium *Synechocystis* sp. (Tabata and colleagues, Cyanobase, DNA Database Japan), as well as one named *pps-1* in *Mycobacterium leprae* (84) has been found. It is notable that in *M. leprae* this ORF contains an intein occupying the central region of the gene. The homology of the bacterial gene to ORF470 is most striking in the 3′ extein (Fig. 4). Finding the same protein-encoding gene in malaria, algae, and bacteria suggests that it has an important and conserved general function; in this connection, it has been noted that 9 of the 12 other inteins known in nature are embedded in three classes of important conserved proteins, namely, vacuolar ATPases, DNA polymerases, and RecA proteins (15). At present, the function of ORF470 is unknown, and it remains to be seen whether the sequences adjoining the bacterial and algal ORFs can afford any clue to the biosynthetic role of this gene. Reith et al. (89b) have made the salient observation that transcription of the ORF is markedly upregulated in *Porphyra purpurea* following exposure of algal cells to light, suggesting a link to metabolic processes.

Immediately 3′ to the three ORFs on the IR_A sector of the malarial plDNA and present on the same DNA strand are three larger genes whose recognition was one of the first clues to the plastid ancestry of the circle. Designated *rpoB*, $rpoC_1$, and $rpoC_2$, they encode subunits β, β′, and β″, respectively, of an RNA polymerase similar to that found in cyanobacteria and chloroplasts and typically not in mitochondria (40). Subdivision of the *E. coli rpoC* equivalent into two genes, $rpoC_1$ and $rpoC_2$, is another plastid-like feature of the diminutive malarial circular genome. Phylogenetic analysis of the *rpoB* gene (38) showed that it has diverged considerably from other plastid sequences, that of the protist *Euglena gracilis* being closest. To date, only a small fragment of the malarial $rpoC_1$ gene has been analyzed phylogenetically (54), and again the *Euglena* plastid sequence showed closest homology. However, the level of conservation of the predicted peptides encoded by *rpoB* and *rpoC* is much lower than for ORF470. For example, *rpoB* of *P. falciparum* encodes a peptide that has only 29% identity with the β subunit predicted from the plastid sequence of spinach (56). Despite this, all the known functional domains of the protein are readily recognized in the predicted malarial peptide, which resembles the plastid type much more than it resembles the bacterial type (Fig. 5). As in the red alga *P. pur-*

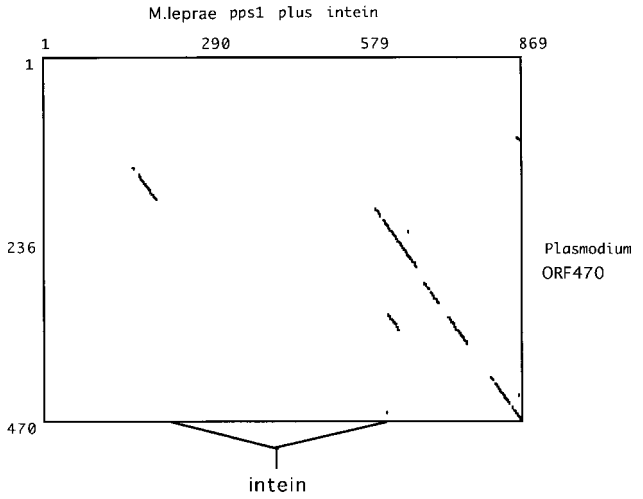

FIG. 4. DOTPLOT comparison of the predicted amino acid sequences of *P. falciparum* ORF470 and *M. leprae pps-1*, showing the position of the intein in the bacterial version of this gene (84).

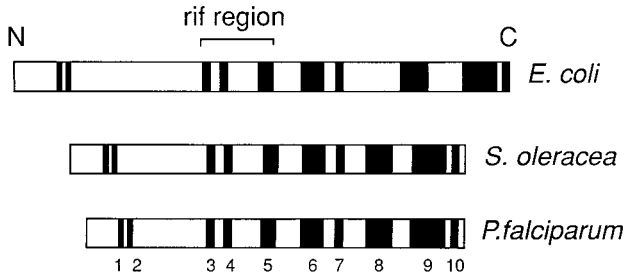

FIG. 5. Schematic showing 10 homologous regions in the beta subunit of the RNA polymerases of *E. coli*, *Spinacea oleracea*, and *P. falciparum* (35-kb circle). The binding site for rifampin (rif) is indicated.

purea (89a) but unlike in most higher plants, the malarial $rpoC_1$ gene does not have an intron.

An intergenic region of 11 nt separates $rpoC_1$ from $rpoC_2$, which codes for the largest subunit of the chloroplast-encoded RNA polymerase. Our sequence contains a −1 frameshift about halfway through $rpoC_2$, with a methionine residue and the remainder of the sequence following immediately after the shift. A structural feature formed by about 600 amino acids encoded in the central region of $rpoC_2$ sequences from higher plants (56) is absent from *E. coli* and the plastid sequences of both *E. gracilis* and *P. falciparum*. The red alga *P. purpurea* (data kindly provided by M. Reith) has a sequence corresponding to about half of it.

As is typical of many plastid but not cyanobacterial genomes, immediately downstream of $rpoC_2$ lies the ribosomal protein gene *rps* 2, whose sequence in the malarial case is not highly conserved. Downstream of *rps* 2, coding switches to the complementary strand, with the direction of transcription pointing away from IR_B, as will be described next.

Single-Copy Region (IR_B Sector)

An ORF lying a few nucleotides 3′ of the trn^T gene marking the end of IR_B was identified by a homology search as the ribosomal protein gene *rps* 4, although the level of predicted amino acid identity to its closest known counterpart, from *Marchantia polymorpha*, was only 27%. As already mentioned, the malarial *rps* 4 sequence shares the same first three codons as ORF470 at the other end of the rDNA palindrome.

Immediately downstream of *rps* 4 lies a cluster of 10 tRNA genes (*his* [H], *cys* [C], *leu* [L], *met* [M], *tyr* [Y], *ser* [S], *asp* [D], *lys* [K], *glu* [E] and *pro* [P]). The leucine gene holds the only intron so far recognized on the circle, located, as with other plastid homologs, within the anticodon (24). The intron is, however, very much shorter than others recorded to date (see the section on codon usage, below). Downstream of the tRNA genes, a series of ORFs encode ribosomal proteins, arranged much as in other plastid genomes. The first ORF in the series has only a low level (19%) of predicted amino acid identity to *rpl* 4, but the likelihood that this gene has been correctly identified is supported by the subsequent ordered series of ORFs corresponding to ribosomal protein genes like those encoded by the *S10*, *spc*, *alpha*, and *str* operons of *E. coli* and chloroplast genomes (Fig. 6). Following *rpl* 4 on the malarial plDNA, we have putatively identified *rpl* 23, *rpl* 2, *rps* 19, *rps* 3, *rpl* 16, and *rps* 17, corresponding to the *S10* operon. None of the predicted peptides is highly conserved, the best being *rpl* 16 with 33% identity to its closest homolog, *Marchantia polymorpha*. A frameshift and a single-base overlap with *rps* 17 leads into sequences corresponding to the *spc* operon. After *rpl* 14, we have tentatively identified *rps* 8, *rpl* 6, *rps* 5, and the small but highly conserved ribosomal protein gene *rpl* 36 (*secX*). Between the last two genes lies an unidentified sequence (ORF91).

Downstream of this *spc*-like operon, another frameshift leads to *rps* 11, a member of the *alpha* operon of *E. coli*. By contrast with the latter, *rpoA* has been deleted from the malarial circle, possibly transposed to the nucleus. It is of interest that unlike *Plasmodium*, the residual plDNA of the parasitic higher plant *Epifagus virginiana* contains a truncated pseudogene for *rpoA* and that this is the only remnant of the *rpo* genes left on that vestigial genome (126).

A few nucleotides after *rps* 11 on the 35-kb circle lies a final pair of ribosomal protein genes, namely, *rps* 12 and *rps* 7, corresponding to components of the *str* operon of *E. coli*. As in

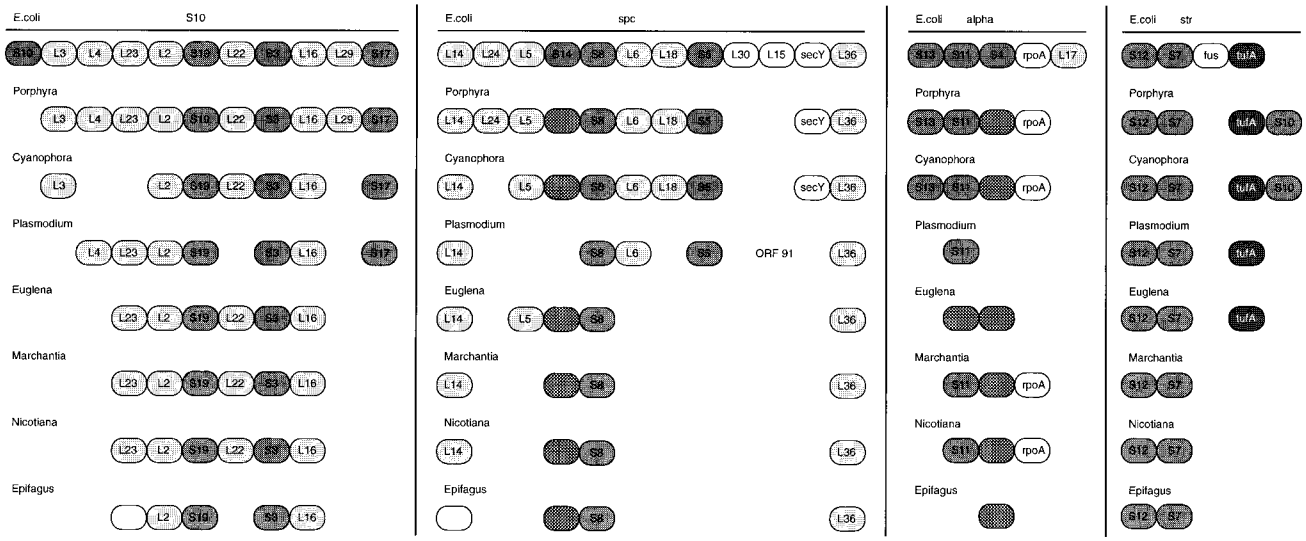

FIG. 6. Ribosomal protein gene operons in *E. coli* compared with those of various plastid genomes. Species include the red alga *Porphyra purpurea*, *Cyanophora paradoxa*, *P. falciparum* (35-kb circle), *Euglena gracilis*, *Marchantia polymorpha*, *Nicotiana tabacum*, and *Epifagus virginiana*. Pseudogenes are indicated by open ovates; transposed gene are indicated by unnumbered stippled ovates. Associated genes include *sec*Y, *rpoA*, and *tufA*. Reprinted from reference 124 with permission.

TABLE 1. Gene content of the 35-kb circular DNA of *P. falciparum*

Class	Genes
rRNA	16S, 23S
tRNA[a]	A^{UGC} C^{GCA} D^{GUC} E^{UUC} F^{GAA} G^{ACC} G^{UCC} H^{GUG} I^{GAU} K^{UUU} L^{UAG} L^{UAA}[b] M^{CAU} M^{CAU} N^{GUU} P^{UGG} Q^{UUG} R^{UCU} R^{ACG} S^{GCU} S^{UGA} T^{UGU} V^{UAC} W^{CCA} Y^{GUA}
Ribosomal proteins	*rps* 2, 3, 4, 5, 7, 8, 11, 12, 17, 19 *rpl* 2, 4, 6, 14, 16, 23, 36
RNA polymerase	*rpoB* $rpoC_1$ $rpoC_2$
Other proteins	*clpC tufA* ORF470
Unassigned ORFs	51, 78, 79, 91, 101, 105, 129

[a] Single-letter amino acid code and anticodon.
[b] Intron.

other algal genomes (53), these precede a *tufA* gene, which encodes the elongation factor Tu (EF-Tu), a G-protein crucial in the elongation step of protein synthesis. Three of the conserved functional domains in the predicted peptide lie in the N-terminal half of the protein and correspond to the GTP-binding site. As in other plastid *tufA* genes, the malarial gene has an insertion which encodes a 10-amino-acid extended loop that may be involved in defining the tertiary structure of the GTP-binding domain (66). It is notable that *tufA* occurs on the plastid genome of many algae but not on that of higher plants (4).

Downstream of *tufA* lie another four tRNA genes: *phe* (F), *gln* (Q), *gly* (G), and *trp* (W). trn^F is on the complementary strand and in this respect is distinct from almost all the other genes on the IR_B arm. Another short tentative ORF, ORF129, then leads to the final large ORF on the IR_B single copy region, provisionally identified as *clpC*, a member of the *hsp100* gene family (124); these genes encode ubiquitous heat shock or stress proteins that act as molecular chaperones with diverse functions (81, 113). Once again, a corresponding gene is present on the plastid genome of the red alga *Porphyra purpurea* but not on the plastids of higher plants. The gene is also absent from the only other fully sequenced plastid genome presently available from a protist, that of *Euglena gracilis* (51). The malarial *clpC* gene is unusual in that only the second of the two ATP binding domains is conserved.

Following the *clpC*-like gene, two tRNA genes encoding alternative codons for *gly* (G) and *ser* (S) are separated by a short region of some 240 nt that contains an unassigned ORF (ORF79). Downstream of trn^S, the 3′ end of another short potential ORF (ORF105) overlaps the *rps* 2 gene on the opposite strand by a few nucleotides, marking the transcription crossover point with the IR_A sector.

The various classes of genes identified on the circle are categorized in Table 1.

Transcription

Transcriptional studies of the plDNA, so far only of intraerythrocytic parasites, are at a preliminary stage. However, transcripts have been found for many of the genes, including all the *trn* genes (86), the rRNAs (36, 37), the *rpo* subunits (40), ORF470, several of the ribosomal protein genes, and both *tufA* and *clpC* (124). Together with preliminary data for the presence of polysomes (92a), these findings provide strong, albeit circumstantial, support for a functional organelle. The levels of transcription of different genes vary widely. The rRNA and *trn* genes are relatively active, while in Northern blots, transcripts for the *rpo* genes and the ribosomal proteins could be detected only in long exposures. In our hands, reverse transcription PCR or RNase protection was the preferred assay for detecting mRNAs. Both the LSU and SSU rRNA transcripts showed evidence of processing from larger precursors, possibly including *trn* genes (36, 37), and we have evidence that the *S10* ribosomal protein operon is transcribed polycistronically (124).

In this connection, the disposition of genes on the two DNA strands may be significant. Starting at the 5′ end of the LSU rRNA gene on IR_A (and moving clockwise on the map in Fig. 2), the LSU rRNA and all the contiguous genes, up to and including *rps* 2, are read from the same DNA strand. Moving anticlockwise from the corresponding site on IR_B, the LSU rRNA gene and, with one exception, all the contiguous genes round as far as the 3′ end of *rps* 2 are located on the complementary strand. The exception is the solitary trn^F gene just downstream of ORF78. The *trn* genes clustered in the two spacer regions of the IR are disposed on both strands as indicated in Fig. 3. The overall arrangement could allow for the occurrence of a few large polycistronic transcriptional units, but this requires further investigation.

Codon Usage

A total of 25 different tRNA genes has been identified, which we believe is sufficient for a minimal translation system, based on the standard code, for all the proteins encrypted by the circle (86). The *trn* sequences conform, for the most part, to the standard secondary-structure cloverleaf specifications. However, trn^G and trn^C both have compensatory changes in the T- and G-loops, and trn^E has ATCC (instead of GTTC) in the T-loop, as is typical of plastids. As mentioned previously, $trn^{L(UAA)}$ contains an intron, located in the anticodon loop. However, this putative class I (self-splicing) intron is very small (only 130 nt compared with those of higher plants, which range from 325 to 2,526 nt) and very divergent in sequence (86). Despite these unusual features, our primer extension analysis indicated that the intron is spliced out precisely from the tRNA transcript (86).

The codon usage of proteins encrypted by the circle is distinctly different from that of either the mitochondrion or the nucleus. As a result of the bias in circle codons attributable to its extreme A+T content, about 80% of the positions in a total of 3,000 codons are accounted for by only seven amino acids (Table 2). Less than 5% of the codons contain a G or C in the third position, and half of these are accounted for by only three codons, AUG (*met*), UGG (*trp*), and AAG (*lys*). Base compositional effects can be extreme; for example, the ORF of *rpoB* is 88% A+T, and 72% of the predicted peptide (a total of 1,024 amino acids) is comprised of just six species of amino acid, each of which has codons made solely from A and T nucleotides. The frequency of both codons and anticodons used by the plastid clearly reflects this biased composition. While at the codon level the shift towards A or T seems paramount, at the level of the anticodons this is not so. Instead, a system has developed which balances the requirement to decode the maximum number of codons with the minimum number of tRNAs against the pressure to use A · T base pairs. As discussed elsewhere (86), there is a notably high percentage of G's in the wobble position of the anticodons, and assuming the usual "wobble" rules, a G · U base pair at the wobble position would allow four codons to be translated by only two different anticodons. Thus, the evidence favors the coevolution of a codon-anticodon recognition mechanism that maintains specificity with a minimum number of tRNAs.

TABLE 2. Frequency of amino acids specified by the nuclear, plastid-like, and mitochondrial genomes of *P. falciparum*

Amino acid	Frequency		
	Nuclear	Plastid-like	Mitochondrial
Ile	7.1	18.0	11.91
Asn	9.7	13.8	3.86
Lys	10.5	13.0	1.46
Leu	8.1	11.5	14.14
Tyr	4.7	10.1	5.74
Phe	3.6	6.0	9.08
Ser	7.0	5.0	9.00
Gly	4.7	3.5	6.43
Thr	4.7	3.0	6.94
Glu	9.0	2.3	1.97
Asp	6.5	2.0	1.89
Val	4.9	1.9	6.17
Arg	2.9	1.8	2.40
Gln	3.4	1.8	1.71
Pro	3.3	1.4	3.94
Met	1.9	1.3	2.66
Cys	1.6	1.2	1.71
Ala	4.1	1.2	3.94
His	2.5	1.0	3.00
Trp	0.4	0.3	2.06

Conservation

Snap-back and cross-hybridization experiments with fragments from the IR of the malarial circle provided evidence for conservation of the rDNA palindrome in two other genera of apicomplexans, namely, *T. gondii* and *Eimeria tenella* (122). These observations confirmed the suggestion of Borst et al. (10) that the cruciform structure they observed in *T. gondii* circular DNA by electron microscopy was derived from palindromic rRNA genes. Hybridization and PCR experiments have also detected sequences corresponding to *rpoB/C*, *tufA*, ORF470, and *trn* in *T. gondii* and *E. tenella*, organized in the same way as in *P. falciparum* (19a). Independent work by others on *T. gondii* (6, 91a) and *Babesia bovis* (44) lends support to the general contention that the circle may be widespread among apicomplexans and highly conserved in its organization, consistent with a monophyletic origin.

Evolution of Apicomplexans—a Plant Connection?

The discovery of the plastid genome in the apicomplexans has stimulated interest in plant-like sequence features of some malarial nuclear genes. For example, an indel apparently characteristic of higher plants was found in the enolase gene of *P. falciparum* (89) while in an analysis of calmodulin, Robson et al. (90) placed the malaria parasite in a monophyletic group with ciliates and plants. Similarly, the observation that the branch point for the malarial histone H2A gene in a dendrogram lay close to that of the pine tree (104) might be taken as another indication of a plant-like relationship. These intriguing observations extend to *T. gondii*, where the predicted amino acid sequence of the heat shock protein hsp30 showed similarity to the conserved C terminus of the 18-kDa heat shock proteins of plants, with the characteristic GVL motif being intact (9). If observations like these are to have a solid basis, they have to be set against compelling evidence, based on classical taxonomy (67) and molecular phylogeny of the nuclear SSU rRNA genes (5, 23, 34, 112), that congruently relate apicomplexans to the dinoflagellate/ciliate clade. Any new evidence that might upset this scenario deserves careful consideration, and at first sight the plastid molecule which has sparked interest in this new aspect of apicomplexan evolution might itself be construed as evidence of this type. However, in our opinion, this is not the case. Far from weakening current received wisdom about the evolution of the apicomplexans, we believe that, if proven, an endosymbiotic algal origin of the plDNA would fit remarkably well with the accepted dinoflagellate-related ancestry of the apicomplexans (112).

Dinoflagellates are a diverse and abundant group of marine or aquatic unicellular protozoa of very ancient origin, fossil traces of their silicaceous envelopes having been found from the Cambrian era. Some species are photosynthetic, and these have brownish plastids (11), which are thought to have been acquired by their habit (or that of a dinoflagellate progenitor) of capturing chromophytic algae. Substantial transfer of algal genes to the nucleus of the host cell must have occurred subsequently to maintain the acquired plastids as organelles (42). On our speculative scenario, this secondary endosymbiotic event served the dinoflagellate well until some 9×10^8 years ago (22, 23), when a single individual is conjectured to have developed a parasitic lifestyle in a polychaete worm (67). The plastid then (if not before) lost its photosynthetic capability and was retained only for some secondary activity. Apart from the presence of the 35-kb DNA, the sole outwardly visible sign of this endosymbiotic history would ultimately be the possession of an organelle with triple or quadruple membranes. It is this feature, characteristic of secondary endosymbiosis (11, 43), which led us to the idea that the multimembraned "spherical body" in the malaria parasite and its analogs in other apicomplexans might be the organellar home of the circular genome (Fig. 1b). An alternative suggestion, that the plastid has only two membranes and lies in a fold of the host cell's endoplasmic reticulum (72), has yet to be confirmed and would perhaps require an alternative explanation from the scheme illustrated in Fig. 7, which is based on the conventional secondary endosymbiosis theory, summarized recently by Palmer and Delwiche (80). Resolution of the membrane topology of the 35-kb DNA-containing organelle has important phylogenetic implications, because algae containing plastids surrounded by four membranes (diatoms, chrysophytes, phaeophytes, haptophytes, chlorarachniophytes, and cryptomonads) probably acquired them by engulfing other eukaryotic algae (12, 111).

Against this background, the jigsaw pieces of traditional taxonomy, molecular phylogeny of nuclear genes, and the occurrence of the residual plastid genome, begin to fit neatly, albeit speculatively, together. The suggestion by Levine (67) that polychaete worms may have been an important intermediate host in the evolution of the apicomplexans adds a further gloss to this construction, because it is generally accepted that polychaete worms were ancestors of the Insecta, and it is thought that apicomplexans like *Plasmodium* were probably well adapted to their insect vectors long before vertebrate hosts appeared (23).

What other evidence can be adduced to support these speculations? It would obviously be helpful if the function of the plastid organelle could be identified (which we discuss below). But perhaps the best clues for wrapping up the origin of the plastid with the apicomplexan radiation will come from molecular phylogenetic analyses of plastid and nuclear genes from dinoflagellates and other forms of algae.

Fossilized or Functional?

Realization that the 35-kb circular DNA is of plastid rather than mitochondrial origin naturally leads us to ask if it is still functional. Just as Wolfe et al. pointed out for the equally enigmatic plastid remnant in the nonphotosynthetic angio-

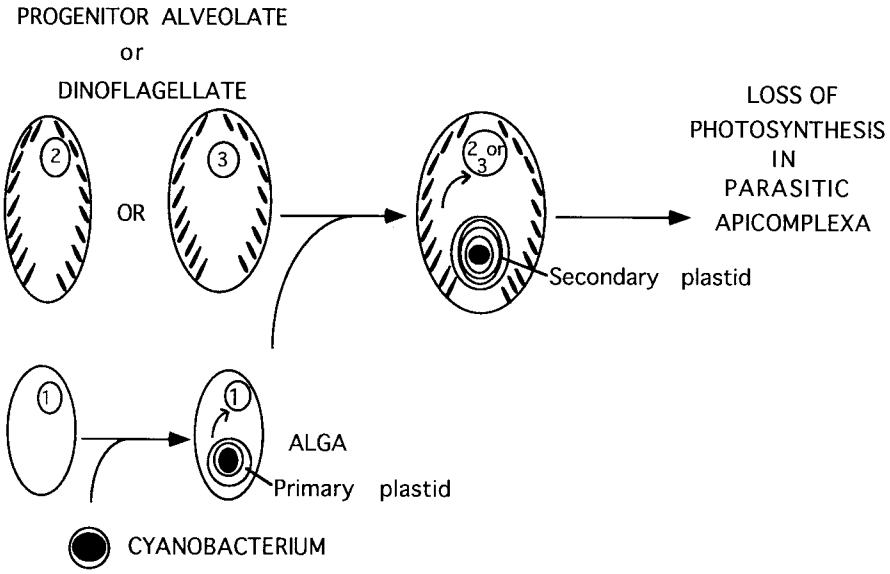

FIG. 7. A hypothetical scheme placing the alveolates with other algae carrying plastids surrounded by four membranes. Such plastids were probably acquired through multiple endosymbiotic events (a eukaryotic alga being engulfed by another) and can be followed by secondary loss of photosynthesis in certain groups (12). The arrows indicate transfer of genes from the primary and secondary endosymbionts to the host cell nucleus (numbered), where they evolved to take over plastid maintenance. Apicomplexans are assumed to be derived from either a progenitor alveolate or a dinoflagellate that adopted a parasitic lifestyle.

sperm *Epifagus virginiana* (126), the skewed nature of the deletions required to produce the malarial plDNA from its presumed algal progenitor (retaining dozens of genes required for gene expression while deleting those directly involved with photosynthesis) is a powerful argument in favor of function. Similarly, the maintenance of large ORFs despite extensive sequence divergence and the conservation of genes across different parasite genera also argue forcibly in favor of function. More direct support is provided by the already noted transcriptional activity of the organelle, and if our preliminary evidence for the presence of organelle polysomes (92a) is confirmed, there can be little reason to think that the organelle is a fossilized, nonfunctional remnant of evolution.

The nature of its role is another matter. Hopes of finding some clue by determining the complete nucleotide sequence of the circle have not proved justified; if there is a key gene that will unlock the puzzle, it is going to be one of the few ORFs which remain unidentified and which, with the exception of ORF470, are rather small. The relatively highly conserved nature of the ORF470 predicted polypeptide (see above) is suggestive and implies that the proteins with which it interacts have evolved similarly and under different selective pressures from, for instance, the plastid's relatively poorly conserved ribosomal proteins. However, despite this, ORF470 may be involved only in plastid housekeeping, and knowing its activity may not bring us nearer to understanding what the organelle actually does. Moreover, we get little help in this connection from the degenerate plastid of *Epifagus*, which has no counterpart of ORF470 and whose genetic makeup has little in common with that of the 35-kb circle, other than the fact that both genomes are replete with genes involved in gene expression.

It seems likely that the apicomplexan plastid organelle contributes to an integral step in eukaryotic intermediary metabolism normally carried out by plastids. Among several possibilities that have been discussed (55, 114), it appears that de novo biosynthesis of heme precursors in the malaria parasite is probably not an answer, since this is carried out by the glycine/mitochondrial pathway rather than by the plastid pathway utilizing glutamate (103).

In contrast to this rather meagre evidence for the function of the malarial 35-kb circle, it has been claimed that a core photosynthetic gene, *psbA*, encoding protein D1 of the photosystem II complex, and also small amounts of protochlorophyllide *a* are present in the apicomplexans *Sarcocystis muris* and *T. gondii* (49). These authors further suggested that the sensitivity of apicomplexans to toltrazuril depends on the interaction of this compound with the D1 protein in a photoreaction center in the parasite organelles. These interesting but provocative suggestions need to be confirmed. However, in our present state of ignorance about the malarial genome, the presence of photosynthetic genes need not be too surprising following the revelation that in dinoflagellates the usual plastid (cyanobacterial) version of the Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) gene (form I) has been replaced with a nucleus-encoded form II Rubisco gene (*rbcL*) of proteobacterial origin (reviewed in reference 79).

THE MITOCHONDRIAL GENOME

The genetic content and sequences of individual genes in the mitochondrial genomes of the few apicomplexans so far studied are moderately well conserved, even though the genomes themselves are quite diverse in their overall physical organization. The most intensively studied are those of the malaria parasites, so much of the following is based on our current understanding of this genus.

Molecular Structure and Genetic Content

***Plasmodium*.** The mtDNAs of the malaria parasites were first detected in *P. yoelii* as variably sized tandem repeats of a 6-kb sequence (108). Identification of their mitochondrial provenance was made only on the basis of their genetic content (2, 107), which was initially seen to comprise genes encoding cytochrome *b* (*cyt b*) and subunit I of cytochrome *c* oxidase (*cox*

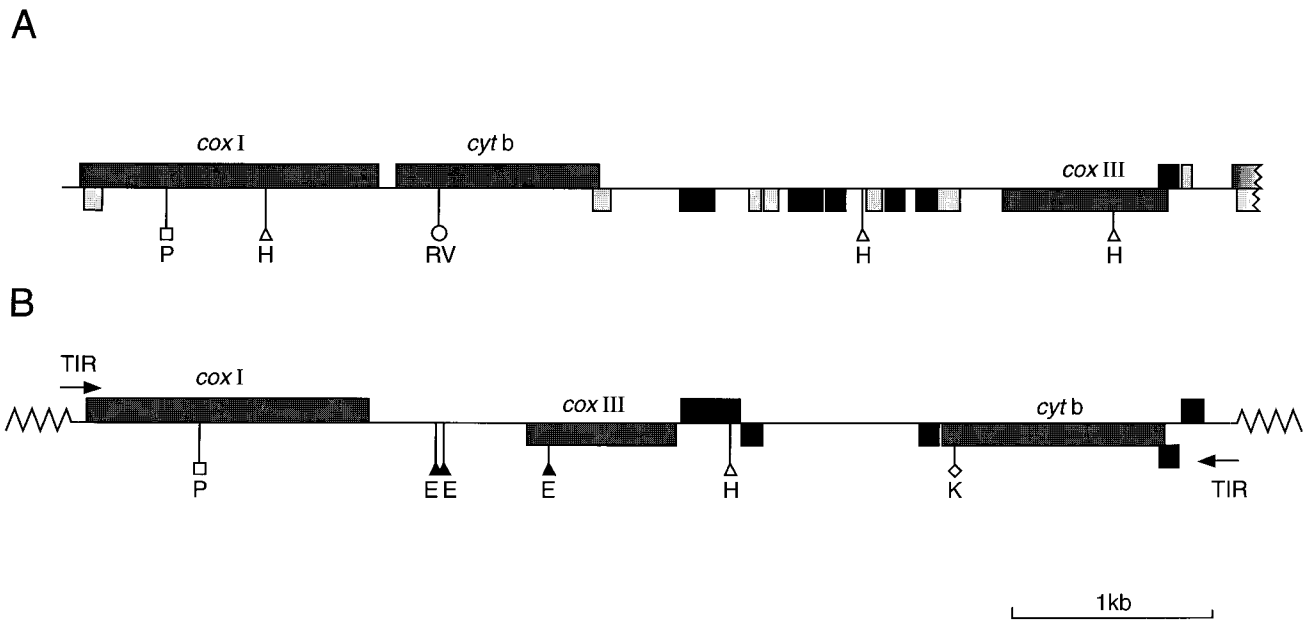

FIG. 8. Diagrammatic genetic maps of mitochondrial genomes. (A) *P. falciparum* (adapted from various sources); (B) *Theileria parva* (adapted from data in reference 62). *cox*, cytochrome oxidase; *cyt* b cytochrome *b*. Lightly stippled blocks indicate fragments of SSU rRNA genes; solid blocks indicate fragments of LSU rRNA genes (SSU rRNA fragments have not been reported in *Theileria*). E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; RV, *Eco*RV. TIR, terminal inverted repeats found near the ends of the *Theileria* molecules. The latter could not be completely sequenced but are nominally about 7.1 kb long. The *P. falciparum* genomes occur mainly as linear head-to-tail tandem arrays (see the text).

I). A third gene, subunit III of cytochrome *c* oxidase (*cox* III), was less well conserved and was found in *P. gallinaceum* by Joseph (60) and later recognized in the complete sequence of the element from *P. falciparum* (25). These three genes comprise the only substantial protein-coding content of the mitochondrial element, although three ostensible small ORFs (none bigger than 151 amino acids, and none generating detectable transcripts) have been reported (109). The genetic layout of the 6-kb element is shown in Fig. 8.

At the time of the 6-kb element's discovery, mtDNAs in general were traditionally expected to comprise uniformly sized circular (or, more rarely, linear) monomers. It is now known, however, that those of several microorganisms, notably filamentous fungi and yeasts, comprise primarily polydisperse linear tandem arrays of a basic genomic unit (70, 71). Thus, malarial mtDNA is not as bizarre as it once seemed but, instead, belongs to an accepted class of microbial mtDNAs. Recent observations on *P. falciparum* (87) have put the finishing touches to this picture by recording the presence of a small number (probably <1% of the mtDNA mass) of circular oligomers including monomers, dimers, and possibly trimers, as well as more complex molecules reflecting the replicative mode of this DNA. There is no evidence that the linear concatemers that make up the bulk of the DNA possess specific terminal or telomere-like sequences.

Two curious features of this element—the smallest mitochondrial genome known—deserve comment. First, unlike most other mitochondrial genomes (*Trypanosoma brucei* is an exception), it encodes no tRNAs. Second, although conforming to the rule that mtDNAs always encode two rRNAs, the genes for these occur as fragments located in an apparently random fashion around the genome, on both DNA strands. These fragments mostly comprise core sequences but are not arranged along the genome colinearly with the usual *E. coli* models, nor are they separated by recognizable introns; rather, they are interspersed with themselves or with the protein-coding genes (25, 26).

Such an arrangement in a mitochondrial genome has been found only once before, in the alga *Chlamydomonas reinhardtii* (8, 46), but in this case all the fragments were encoded on the same DNA strand. Disrupted genes for cytoplasmic rRNAs also occur in *Crithidia*, *Trypanosoma*, and *Euglena* spp. (116), and it has been suggested that such fragmentation may be a primitive feature, usually lost in later evolution (8). There is no evidence that the malarial mitochondrion generates full-sized rRNA molecules. However, this does not militate against its being functional; *Chlamydomonas* has functional mitochondrial ribosomes, and there is ample evidence that fragmented cytoplasmic rRNAs can give rise to ribosomes that work (see reference 95 and references therein). On the other hand, in *Chlamydomonas* mitochondria, the gaps in the ribosomal structure are all outside core sequences, which is not true in the malarial case, and it is not yet clear that all the core rRNA fragments expected on the basis of the *E. coli* secondary-structure models are encoded (26, 29). As small ribosomal size is not uncommon amongst protists (45), this feature alone does not rule out a functional role for the ribosomes.

Theileria. The finding of cross-hybridization of total DNA from *Theileria parva* with the 6-kb element of *P. gallinaceum* (61) gave the first indication that *Theileria* carries a comparable multicopy DNA. However, it differs from the malarial genome in consisting of a single-sized linear molecule, not tandemly repeated. Hall et al. (50) reported the *T. annulata* version as a 6.5-kb multicopy sequence, which they initially supposed had a chromosomal origin, but correctly identified its mitochondrial provenance when they found it encoded a gene for *cyt b* (73). A more detailed study of the comparable element from *T. parva* (62) showed that it had essentially the same genetic content as the malarial version, although, as illustrated in Fig. 8, there are significant differences in gene order between the

two genomes. A more substantial difference is that the *T. parva* version comprises monomeric genome-sized linear molecules with terminal inverted repeats indicative of telomeres, while its malarial counterpart consists of polydisperse linear tandem arrays without telomeres (108). The origin of such a major structural divergence between genera of the same phylum is not known, but one might speculate that an ancestral molecule of the *Theileria* type could have undergone successive tandem duplications, thereby generating arrays of the malarial pattern. Rearrangements of gene order and loss of telomeres would also have been needed to complete a transition from one type to the other. Because of the difficulty of sequencing the ends of the *T. parva* molecule, the estimate of its size, 7.1 kb, is necessarily approximate, only 5,895 bp having been sequenced. As in *P. falciparum*, the rRNA genes were represented by dispersed fragments. Only sequences corresponding to domains IV and V of the *E. coli* LSU rRNA secondary-structure model have been identified, with the order within the element differing from that found in *Plasmodium*. A search for sequences corresponding to the α-sarcosin loop (domain VI) identified what might be a related sequence, with the central GAGA motif replaced on the *T. parva* element by GTAA (62). Small RNA fragments presumed to correspond to rRNA transcripts could probably function in a ribosome, since in this case the supposed breaks between the fragments fall outside core rRNA regions, but there are probably many more rRNA gene fragments to be detected.

Toxoplasma. mtDNA of *T. gondii* has yet to be isolated and sequenced. This is largely due to technical problems arising from the presence in this organism of fragments derived from characteristic mitochondrial genes (*cox* I and *cyt b*) integrated into nuclear chromosomes (78). It seems unlikely that these nucleus-encoded fragments, which are bounded by inverted or direct repeats, are functional, but the nearest published sighting of what may be the true mtDNA in *Toxoplasma* was the observation in CsCl-Hoechst gradients of a minor band a little less dense than the main band of nuclear DNA (61).

Babesia. A radioactive probe made from the mtDNA of *P. gallinaceum* detected a fragment of around 7 kb in *Hin*dIII-digested genomic DNA of *Babesia microti* (61). It is possible that this was the counterpart of the multicopy 9- and 7.4-kb molecules recorded in undigested genomic DNA of different *Babesia* species (59, 94). However, in neither of these two cases was sequence or hybridization data available to confirm the identity of the molecules, and no direct experimental evidence was presented by Jasmer et al. (59) to support their contention that the molecule they described occurred as a covalently closed circle, ca. 20 kb in contour length.

Gene Expression

It is well established that the mtDNA of malaria parasites is transcriptionally active (102, 108). Evidence for several small polyadenylated transcripts ranging from 0.3 to 1.6 kb was accompanied by the caveat that the A+T-rich nature of the DNA might be responsible for its retention on oligo(dT) columns—this was (and is still) the only indication of their polyadenylation. Even though detailed knowledge of the mechanism of transcription is lacking, the finding of transcripts underpins a general belief that the malarial organelle is functional in the erythrocytic stages. The three protein-coding genes (*cyt b*, *cox* I, and *cox* III) give rise to transcripts commensurate with their size (102), consistent with evidence suggesting that their polypeptide products are present and functional (26). It is not clear if these transcripts are processed from a larger precursor, although 5-kb transcripts of both strands were found in *P. gallinaceum*, in addition to transcripts of 1.8 and 1.2 kb and <300 nt (2). Two transcripts slightly larger than 2.3 kb were reported in *P. falciparum*, but no functional attribution was made (109). The whereabouts and nature of promoters in this genome are unknown.

Much interest attaches to the fragmented nature of the rRNA genes of apicomplexans. The small transcripts (<300 nt) seen by all authors were apparently generated from these coding sequences, and in the *Plasmodium* species that have been most intensively scrutinized, no molecules corresponding to the usual 16S or 23S rRNAs which would be generated by ligation of these smaller fragments have been detected (26). This situation does have a precedent; in *Chlamydomonas*, the small fragmentary transcripts of mitochondrial rRNA genes are not ligated to form "normal"-sized rRNAs, and it is assumed that they are held together in the ribosomes by noncovalent interactions. Further study is required to confirm if this is the case in the malaria parasites.

In an analysis of transcription of the mtDNA in the erythrocytic cycle (27), it was found that, relative to nuclear SSU rRNA, transcripts of the mitochondrial rRNA gene fragments are about equally abundant throughout the cycle. In contrast, transcripts of the protein-coding genes were barely detectable in the ring stage but increased sharply in amount during schizogony. Since the abundance of transcripts of the nuclear rRNA genes during the erythrocytic cycle has not been studied in detail, the absolute cellular abundance of these transcripts needs further study.

An ATG near the 5′ end of the *cyt b* gene is thought to be the initiation codon (26), but neither of the other two protein-coding genes is similarly endowed. Sequence determination of the 5′ ends of their transcripts has shown them to be identical to the genomic sequences, so there is no evidence for RNA editing or other phenomena that might endow these with AUG initiation codons, and possible alternatives have been discussed (26). In all other respects, the standard genetic code appears to apply to these genes.

Codon usage of the mtDNA genes, necessarily based on only the three polypeptides it encodes, differs from those of both the nuclear and plastid-encoded polypeptides (Table 2).

The only nonmalarial apicomplexan mtDNA to have been analyzed at all in regard to gene expression is that of *Theileria parva* (62). A limited Northern blot analysis revealed several transcripts ranging in size from <360 nt to nearly 1.8 kb. Transcripts of the fragmented rRNAs were not identified, but examination of a cDNA library made from poly(A)$^+$-enriched RNA led to the isolation of a clone for *cyt b* that was nearly full length and was identical to the genomic copy. In this organism, translation of both *cyt b* and *cox* III apparently starts at AUG codons, whereas the *cox* I ORF begins with an AGU. It is not known if initiation starts at this unusual codon, or whether the mRNA is posttranscriptionally modified (62).

Replication

The timing of replication of mtDNA in blood cultures of *P. falciparum* is well established, at least in outline. It has been known for many years that bulk DNA synthesis starts in blood stage culture in late trophozoites (47, 57, 91, 99), not long before the onset of the nuclear divisions that mark the start of schizogony. It is now known that both mtDNA and nuclear DNA start replicating together (87, 99).

Little is known about the enzymology of malarial mtDNA replication. There is evidence that malaria parasites carry at least one DNA polymerase resembling DNA polymerase γ in its drug sensitivities (14), and in vitro an aphidicolin-resistant

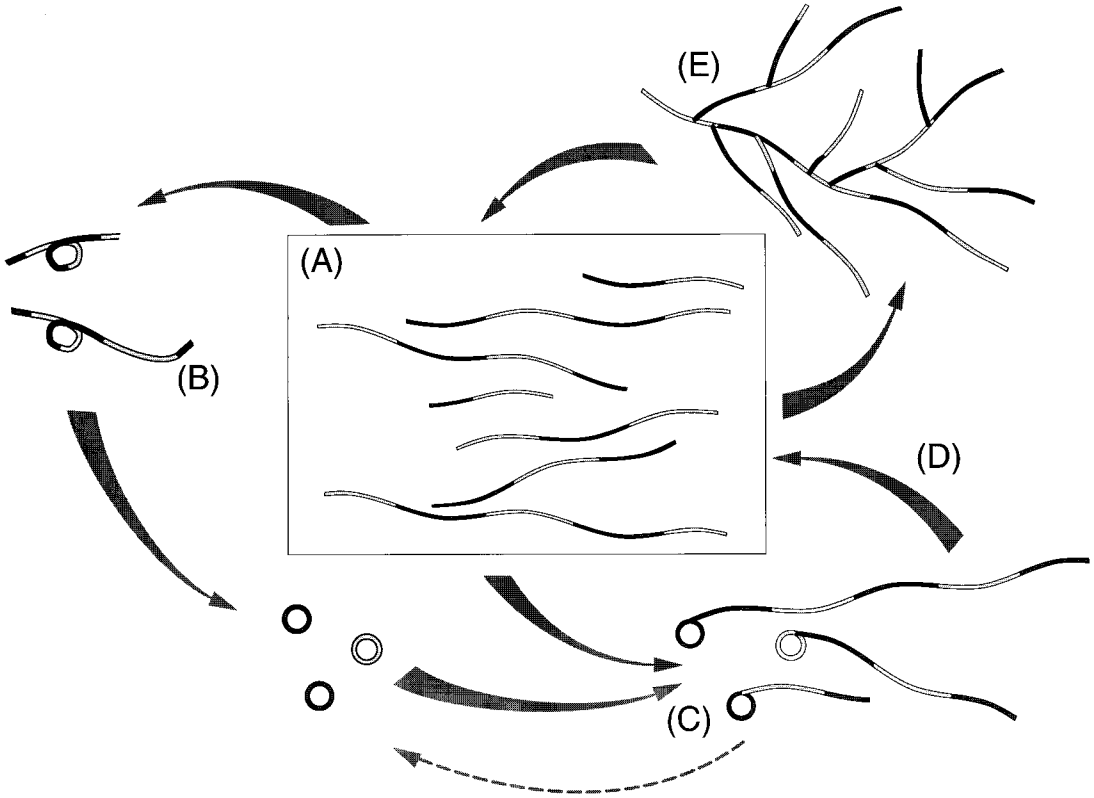

FIG. 9. Schematic of events involved in replication of mtDNA in *P. falciparum* (87). The mtDNA of nonreplicating erythrocytic parasites (A) comprises around 20 copies of the 6-kb genome in a few linear tandem arrays of one to five repeats in length. The unprotected termini have a 3′ unreplicated overhang. Replication is accompanied by various recombination activities. Intramolecular recombination (B) produces some circular forms. They enter a rolling-circle replication process that may depend on homologous recombination between circles and the termini of linear molecules (C). This generates lariats that yield linear concatemers (D) and possibly some circles (dotted arrow). Finally, during the mitochondrial S phase, a major recombinational activity forms complex networks (E) that are processed to yield more linear concatemers.

enzyme was shown to be highly sensitive to the nucleotide analog (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) (99). However, although HPMPA effectively arrested parasites in schizogony, the replication of both the organelle DNAs in vivo was unaffected by the inhibitor, so it is not clear if the same polymerase is responsible for replication of either of the organelle DNAs.

Recent studies in our laboratory (86) have begun to unravel the basic replication mechanics of malarial mtDNA, and, as might be expected from its overall organization, the mechanism is both complex and surprising. The use of two-dimensional gel electrophoresis and electron microscopy has revealed that the parasite mtDNA replicates in a manner that is reminiscent of mechanisms used by some bacteriophages and bacterial plasmids (Fig. 9). The linear tandem arrays that form the bulk of the mtDNA enter into multiple recombinational interactions with other molecules in the pool, forming complex aggregates like those seen in replicating phage T4 particles. At the same time, some circular molecules undergo rolling-circle replication, another process associated with bacteriophages and some bacterial plasmids, producing long linear concatemers of the 6-kb genome.

This is, to our knowledge, the first time that phage-like replication mechanisms have been recognized in mitochondria, although some yeasts and fungi have been shown to have similarly structured mtDNA, while extensive recombination of mtDNA molecules is a recognized feature of *Saccharomyces cerevisiae* (97, 117) and may also occur in *Schizosaccharomyces pombe* (52). It will be of interest to explore the enzymology of this unusual mode of replication, and because of its genetic flexibility, brewer's yeast may provide a useful model for analysis of the malarial system.

ANTIBIOTIC INDICATORS OF FUNCTION

Potential nucleotide target sites for antibiotics have been pointed out in the sequences of the extrachromosomal DNAs of *P. falciparum* (29, 37, 115). However, malaria parasites were found to be resistant to a number of these agents, notably aminoglycosides, although they were sensitive to inhibitors of 70S ribosomes (41). One of these, tetracycline, has had useful clinical application as an antimalarial agent (16) and is believed to act on the mitochondrion, reducing the activity of dihydroorotate dehydrogenase, an important mitochondrial enzyme central to pyrimidine biosynthesis (85). (The possibility that tetracycline also acts on the putative plastid compartment has not been explored.) By contrast, the mitochondrion of *Toxoplasma gondii* could not be positively identified as the lethal target of macrolide antibiotics (6), and the possibility was mooted that the plastid might be the actual target. An increase in sensitivity to clindamycin—a macrolide proposed to act on the putative plastid (6, 83)—was found in a mutant selected for resistance to the antimitochondrial drug atovaquone (106). As the mitochondrial inhibitors atovaquone and myxothiazole induce a switch from the tachyzoite to bradyzoite stage of *T. gondii*, Tomavo and Boothroyd (106) have proposed

that products of both mitochondrial and plastid organelles may be involved at some step in the regulation of this differentiation pathway. The delayed action of clindamycin on *T. gondii*, where the effects become apparent only upon invasion of a new cell, is remarkable and as yet unexplained (30).

The presence of the *tufA* gene on the plDNA has led us to investigate antibiotics known to act on the elongation cycle in prokaryotic ribosomes (101a), and we found different levels of antimalarial activity with three such compounds, i.e., kirromycin, which prevents the release of EF-Tu after GTP hydrolysis (100), fusidic acid, which has been found previously to have antimalarial activity in blood cultures (7), and thiostrepton, which binds to nucleotides A_{1067} and A_{1095} in the 23S rRNA of *E. coli*, preventing the release of EF-G (92, 93, 105). While the sites of antimalarial action of these compounds remain to be defined, it is notable that the nucleotide corresponding to A_{1067} is conserved in the pl 23S rRNA of *P. falciparum* but is substituted by G in both its nuclear and mtDNAs (115). Likewise, we note that A_{1095} is conserved in the pl 23S rRNA but not in the appropriate mtDNA fragment. These results suggest that several consecutive steps of organellar protein synthesis in the parasite might be inhibited with antibiotics. However, more work is required to unravel the sites of action because of the complication that two target organelles, the plastid and the mitochondrion, are available.

Identification of the *rpoB* gene on the plDNA, coupled with the sensitivity of malaria parasites to rifampin in blood cultures (41) suggested the possibility of a causal relationship (40). However, this again has not been proved, and it remains just as likely that rifampin exerts its activity at some other site (101). Despite such uncertainty, it is not without interest that this drug had significant antimalarial activity, although without effecting a cure, in a clinical trial of patients infected with *P. vivax* (88). The related drug rifabutin has shown promising activity in vitro against *T. gondii* (3, 77), especially when used in combination with other drugs whose action it potentiates.

The effects of amino acid substitutions found in the natural variant of *cyt b* in *Plasmodium* have been discussed in a speculative manner in relation to sensitivity to inhibitors such as hydroxynaphthoquinones and 8-aminoquinolines (109). In addition, the relative resistance to standard mitochondrial inhibitors such as antimycin was attributed to single-amino-acid changes in the Q_i center ($F_{225} \rightarrow L$ and $K_{228} \rightarrow L$; numbering for *S. cerevisiae* [74]). However, a simple explanation for the natural resistance of *P. falciparum* to myxothiazole, acting in the Q_o center, was not immediately apparent from the sequence. Further exploration of this point might be of interest as the level of resistance has been enhanced still further by selecting lines of *P. falciparum* with a classical point mutation for myxothiazole resistance in *cyt b* ($G_{137} \rightarrow S$ [101a]).

In summary, the molecular genetic studies of mtDNA carried out so far are consistent with the limited biochemical functions proposed for the malarial mitochondrion (31, 32), but they have added little to our understanding of mitochondrial metabolism, nor have they had a practical impact on the devising of novel inhibitors. It is to be hoped that the transition can be made from the available parasite sequence information to useful predictive work based on existing models of molecular structure, such as that for *cyt b* (19) and cytochrome *c* oxidase (58). Work of this type has yet to be reported.

CONCLUSIONS

The functional importance of the mitochondrion in apicomplexans is only beginning to be appreciated, despite a long-standing awareness of its potential as a chemotherapeutic target. This organelle has largely eluded the efforts of biochemists either to purify it or to dissect out its biochemical pathways, enhancing the value of the recent molecular genetic studies of mtDNA. These studies have revealed an unusually limited coding potential, but, more interestingly, they have provided sequence information for highly conserved proteins like cytochrome *b*, which participate in complex III activities. The molecular analysis of this and the cytochrome oxidase subunits encoded by the mtDNA now needs to be carried forward to the structural level.

The surprising finding of the fragmented mitochondrial rRNA genes poses other issues, perhaps of interest to a wider church—those concerned with ribosome evolution and function. In its bacteriophage-like mode of replication, the mtDNA of the malaria parasites is not entirely atypical, since it has clear counterparts in some yeasts and fungi, but this too raises interesting questions about the evolutionary origin of these genomes.

Similar questions are also underlined by the structural diversity that is beginning to be apparent in the mtDNAs of different apicomplexans. However, in the broad context of other microbial eukaryotes, such diversity is clearly not a quirk only of the apicomplexans (33, 116). Although making little obvious sense with regard to the supposed monophyletic origin of apicomplexan mtDNA, it presumably reflects variations in the selective pressures encountered by organisms evolving in different environmental niches.

The full implications of the proposed plastid origin of the circular DNA of apicomplexans have still to be worked out. The photosynthetic ancestry of these organisms had not been foreseen, although descent from a dinoflagellate/ciliate clade was evident from earlier phylogenetic studies of nucleus-encoded rRNA genes. Unfortunately, the high evolutionary rate of the plDNA has made it difficult to reach clear conclusions about its source, and work remains to be done here. Comparative studies of the plDNA within the Apicomplexa should determine whether the present genetic organization of the molecule has a long history, as we propose. The fact that the *P. falciparum* ORF470 gene has been traced back to gram-positive bacteria may ultimately allow us to determine the function of this intriguing gene in a more tractable system.

Of immediate practical interest, the presence within the parasites of the novel organelle signalled by the 35-kb DNA may provide an explanation for the mode of action of some antimicrobial agents or may even offer the possibility of devising new specific inhibitors. Such predictions must be tempered with caution, however, as transcription of plastid genes, in the blood forms of malaria parasites at least, appears to be at a low level, and organellar proteins have yet to be described.

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ADDENDUM IN PROOF

Kohler et al. (Science, in press) have demonstrated localization of the 35-kb DNA, as well as rRNA and transcripts of one plastid-encoded ribosomal protein, in the multimembraned Golgi adjunct of *T. gondii*. Phylogenetic analysis of the *tuf* gene on the 35-kb DNA confirmed its placement with other plastids, particularly those of "green" lineages.

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